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Molecular characterization and antibiotic susceptibility of *vibrio cholerae* isolates in Southern Ghana

BY

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DECLARATION

I declare that this thesis is the result of my personal work. Apart from references to the works of other people's work which I have duly referenced, this thesis is the result of my own study done at, Department of Clinical Microbiology, KNUST, under the supervision of Dr Patrick Feglo. This work has neither in part nor in whole been presented anywhere for another Master's Degree or of any kind.

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DEDICATION

This work is dedicated to my parents Mr &Mrs Sewurah, my husband Mr John Adekpo and my siblings.

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I am heavily indebted to express my profound gratitude to many who had contributed in diverse ways to make this project a success. My first and foremost thanks goes to the Almighty God for his guidance, protection, direction and providing me with all that it took to come out with project. I wish to express my sincere gratitude to my hard working supervisor Dr Patrick Feglo for his patience and time spent in correcting my mistakes and providing me with the necessary information to come out with this work. I am forever grateful to Dr T.B Koffie (HOD), the lecturers and the entire staff of Clinical Microbiology Department, KNUST.

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ABBREVIATIONS

NPHRL	National Public Health and Reference Laboratory
KATH	Komfo-Anokye Teaching Hospital
PFGE	Pulse Field Gel Electrophoresis
ICE	Integrative and Conjugative Elements
CT	Cholera Toxin
TCP	Toxin Coregulated Pilus
LPS	Lipopolysaccharide
CFTR	Cystic Fibrosis Trans-membrane Conductance Regulator
VPI	Vibrio Pathogenicity Inland AC
	Adenylate Cyclase
PCR	Polymerase Chain Reaction
TCBS	Thiosulfate-Citrate-Bile Salts-Sucrose Agar
GAR	Greater Accra Region

ABSTRACT

This study was carried out to determine the presence or absence of virulence genes in *Vibrio cholera* isolates in Ghana, and also to determine susceptibility of the isolates to commonly prescribed antibiotics for the treatment of cholera in Ghana. Forty (40) *Vibrio cholera* isolates obtained from the National Public Health and Reference Laboratory, Korle-Bu and Komfo-Anokye Teaching Hospital were used for the analysis. All 40 isolates were susceptible to tetracycline and ciprofloxacin but resistance was demonstrated in doxycycline, ampicillin cotrimoxazole, and erythromycin. Thirty-nine of the isolates showed presence of genes coding for virulence (toxin coregulated pillus and cholera toxin gene) whiles SXT element was also present in 39 *Vibrio cholera* isolates. Pulse field gel electrophoresis on the isolates showed clusters similar to strains from DR Congo, Togo,

Cameroun and Ivory Coast.

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CHAPTER ONE

1.0 INTRODUCTION

Cholera is an acute diarrhoeal ailment caused by toxigenic *Vibrio cholerae*. The disease can lead to death within hours if left untreated. Two important serogroups O1 and O139 can cause recurring outbreaks reaching epidemic and pandemic levels (Luo et al., 2013). However non O1 and non O139 serogroups have also been linked with cholera-like illness (Luo et al., 2013). Cholera remains a major public health concern particularly in developing countries where there is absence or insufficient source of clean water, poor sanitation and basic hygienic facilities(Akoachere & Mbuntcha, 2014)

Cholera cases are estimated to be 1.4 to 4.3 million with 28,000 to 142,000 deaths annually worldwide (Republic & Est, 2014). More than forty years after its emergence, the disease still remains a serious public health problem particularly in Africa. Its occurrence is seen in the Great lake of the Rift Valley and the basin of lake Chad but most especially in Northern Nigeria, Northern Cameroun and Chad, Coastal regions in West Africa between Guinea Bissau and Ghana and the East African coast between Tanzania and Mozambique (Mengel, 2014). Cholera in Africa is often demonstrated by recurrent outbreaks and high incident of death (Mengel, 2014). Between 1970 and 2012, 3,316,201 alleged cholera cases were reported to WHO in African countries representing 40% of 7,199,044 cholera cases reported globally(Mengel, 2014). In 2013, an overall of 129,064 cases were registered from 47 countries, out of which 2,102 deaths were recorded from 26 countries giving a case fatality rate (CFR) of 1.63%. Cases were reported from 22 African countries, 14 Asian countries, 2 European countries, 8 American countries and 1country from Oceania.

(Republic & Est, 2014). Out of the 26 countries that reported death from cholera, 17 were from the Africa continent accounting for 1,366 deaths with a case fatality of 2.43%, while Americans, Dominion republic and Haiti reported 635 deaths with a CFR of 1.04% (Republic & Est, 2014). Between June 2014 and January 2015, West and Central Africa cholera outbreak claimed 1,683 death, over 91,361 reported cases and case fatality rate of

2% in 11 countries which is 3 times more than that in 2013. (Wikipedia accessed February 25th 2015)

Cholera was first recorded in Ghana in 1970 at the Kotoka International Airport from a Togolese national travelling from Guinea (Pobee& Grant, 1970). Two of the worst hit places were the fishing communities of Akplabanya (in the then Ada District) and Nyanyano (Winneba district) (Pobee & Grant, 1970). Cholera in these areas were "sneaked in" by relatives of dead Ghanaian fishermen from Togo and Guinea (Pobee & Grant, 1970). Cholera in Ghana is now endemic and greatly enhanced by improper food hygiene, sanitation, environmental and waste disposal(Ofori-Adjei & Koram, 2014). The disease is an urban problem with great effect on the urban poor(Ofori-Adjei & Koram, 2014). Deprived environmental conditions such as lack of potable drinking water and severe challenges in waste clearance are the results of unregulated urban growth (Ofori-Adjei & Koram, 2014). The 2014 cholera outbreak in Ghana started in June and a total of 28,975 cases were reported with 243 deaths (CFR of 0.8%). All 10 regions of the country reported cholera outbreak by the end of 2014 which includes 130 out of the 216 districts. (WHO, 2015). The Greater Accra region alone contributed about 74% representing a total of 20,197 cases as compared with the Upper West region which recorded 36 cases (WHO, 2015). As of 20th September 2015, 34

districts in 9 regions recorded a cumulative total of 683 cases with 10 deaths (CFR of 1.5%) (WHO, 2015).

1.1 PROBLEM STATEMENT

Ghana suffered its first cholera outbreak in the year 1970 and the country has been experiencing epidemic outbreak of cholera ever since. Ghana has not recorded such a great number of cholera cases for about thirty years now except the 2014 cholera outbreak which recorded 28,975 cases with 243 deaths by the end of 2014(WHO,2015). This sudden rise in the total number of cases reported in the year 2014 compelled the conduct of this study to determine whether it is a totally new strain or an old strain, already known to be circulating in Africa.

1.2 JUSTIFICATION

The toxigenicity of a *Vibrio cholerae* strain is determined by its ability to produce cholera toxin encoded by CtxAB genes and toxin coregulated pilus encoded by the vibrio pathogenicity island. However non-01/0139 also exhibits these virulence factors without causing epidemic threat. For diagnosis of an infectious outbreak, early detection and identification of the causative microorganism is very crucial. Hence the need to differenciate between epidemic *Vibrio cholerae* 01 and 0139 strains and other *Vibrio cholerae*. The recent cholera epidemic in Ghana has necessitated an investigation into the cause of this widespread outbreak that has claimed so many lives. This investigation would establish whether it is a new and more virulent strain or the same old strains. It would also reveal whether different multiple strains are responsible for the outbreak. It would also reveal the antimicrobial susceptibility pattern of the isolates.

1.3 AIM

 The aim of this study was to determine the molecular characteristics and the antimicrobial susceptibility profile of *Vibrio cholerae* isolates obtained in the 2014 outbreak in Ghana.

1.4 OBJECTIVE

The specific objectives of the study was

- To isolate *Vibrio cholerae* from rectal swab
- To determine the serotype of the *Vibrio cholerae* isolates
- To determine the antimicrobial susceptibility pattern of the *Vibrio cholerae* isolates
- To determine the presence of the two genes encoding *Vibrio cholerae* virulence and SXT resistance gene by Polymerase Chain Reaction(PCR)
- To fingerprint the *Vibrio cholerae* isolates and to compare the genotypes to other isolates circulating in Africa by PFGE

CHAPTER TWO

LITERATURE REVIEW

2.1 DEFINITION OF CHOLERA

Cholera is a severe diarrhoea illness caused by a bacterial infection of the intestine (Lamond & Kinyanjui, 2012). *Vibrio cholerae* of type O1 or O139 is responsible for this disease (Lamond & Kinyanjui, 2012). About 10-20 percent of the acute watery diarrhoeal cases are associated with vomiting (Lamond & Kinyanjui, 2012). If persons with the sickness are not instantly and effectively treated, the loss of huge volumes of fluid and salts through diarrhoea and vomiting leads to severe dehydration and death within hours (Lamond and Kinyanjui, 2012).

2.2 CHOLERA EPIDERMIOLOGY

In the earlier days it was an infection common throughout the world, but the epidemic is now mainly to developing countries in the tropical zone and subtropics(Qadri et al., 2005).

This disease is prevalent in Africa, portions of Asia, the Middle East, and South and Central America (Qadri et al., 2005). Outbreaks typically occur when war or civil instability disturbs public hygiene services (Qadri et al., 2005). Natural disasters like earthquake, volcanic eruptions, tsunami, landslides and floods all add to outbreaks by distracting the normal balance of nature (Qadri et al., 2005).

2.3 HISTORY OF CHOLERA

History has it that there have been seven pandemics of cholera (Reeves & Lan, 1998).

Records of cholera goes back to more than 2000 years in India and China (Reeves & Lan, 1998) but appeared in Europe in 17th century(1817-1823) which was later taken as the first of the seven recognized pandemics of cholera (Barua,1992). It was during the 5th pandemic that *Vibrio cholerae* was described and recognized as the causal agent of cholera but strains were studied in detail only in the sixth and seventh pandemic with the sixth pandemic ending in 1923((Reeves & Lan, 1998). El Tor biotype of *V. cholerae* was the cause of the seventh pandemic. It originated in Indonesia in 1961, extended through Asia, and got to

Africa in 1970. It affected the West African countries of Guinea, Sierra Leone, Liberia, Ghana and Nigeria and spread along rivers and trade routes (Gaffga et al.,2007). In 1991, it appeared suddenly in Latin America (Reeves & Lan, 1998). In between the sixth and the seventh pandemic (1923-1961), there was a period of 38 years in which cholera occurred as local outbreaks instead of spreading as pandemics. Strains of *V. cholerae* O1 isolated within this era were denoted as the pre-seventh pandemic El-Tor strains (Nair et al., 2006).

The second biotype of *V. cholerae* O1 also known as the classical biotype, is now rarely seen. This biotype has been the cause of the fifth and sixth pandemics and is alleged to have been linked with the previous pandemics as well, though there is no concrete proof

(Samadi et al., 1983). The classical biotype has been entirely displaced globally, except in Bangladesh where it resurfaced in epidemic extents in 1982 (Samadi et al., 1983). In the 19th century, cholera spread throughout the world from its initial origin in Bangladesh and West Bengal(Mitra et al., 2001;Karaolis et al., 1998) resulting in loss of lives across all continents, particularly Africa and Asia. There was another epidemic in Bangladesh in 2004 that was caused by post flooding(Qadri et al., 2005). More than 17, 000 cases were involved during the outbreak, with the isolation of *Vibrio cholerae* O1 serotype Ogawa & Inaba

(Qadri et al., 2005). Cholera is now rampant in lots of countries and current studies have shown that global warming creates the right atmosphere for the microorganism to thrive (Lipp et al., 2002).

2.4 CHOLERA IN GHANA

Cholera was first recorded in Ghana in 1970 at the Kotoka International Airport from a Togolese national who was travelling from Guinea (Pobee & Grant, 1970). Later that year, two affected areas in Ghana were identified, which were the fishing communities of Akplabanya located in the then Ada District and Nyanyano situated in Winneba District. There after cholera was isolated from many patients in many parts of Ghana and the disease has become endemic (Oteng-Ababio, 2014).

Ghana has always been ranked among the most affected countries in the African continent. An annual average of 3,066 (range: 50-10,628) cases of cholera were recorded between the year 2000 and 2013 with an overall case fatality rate of 1.7 % (Eibach et al., 2016).

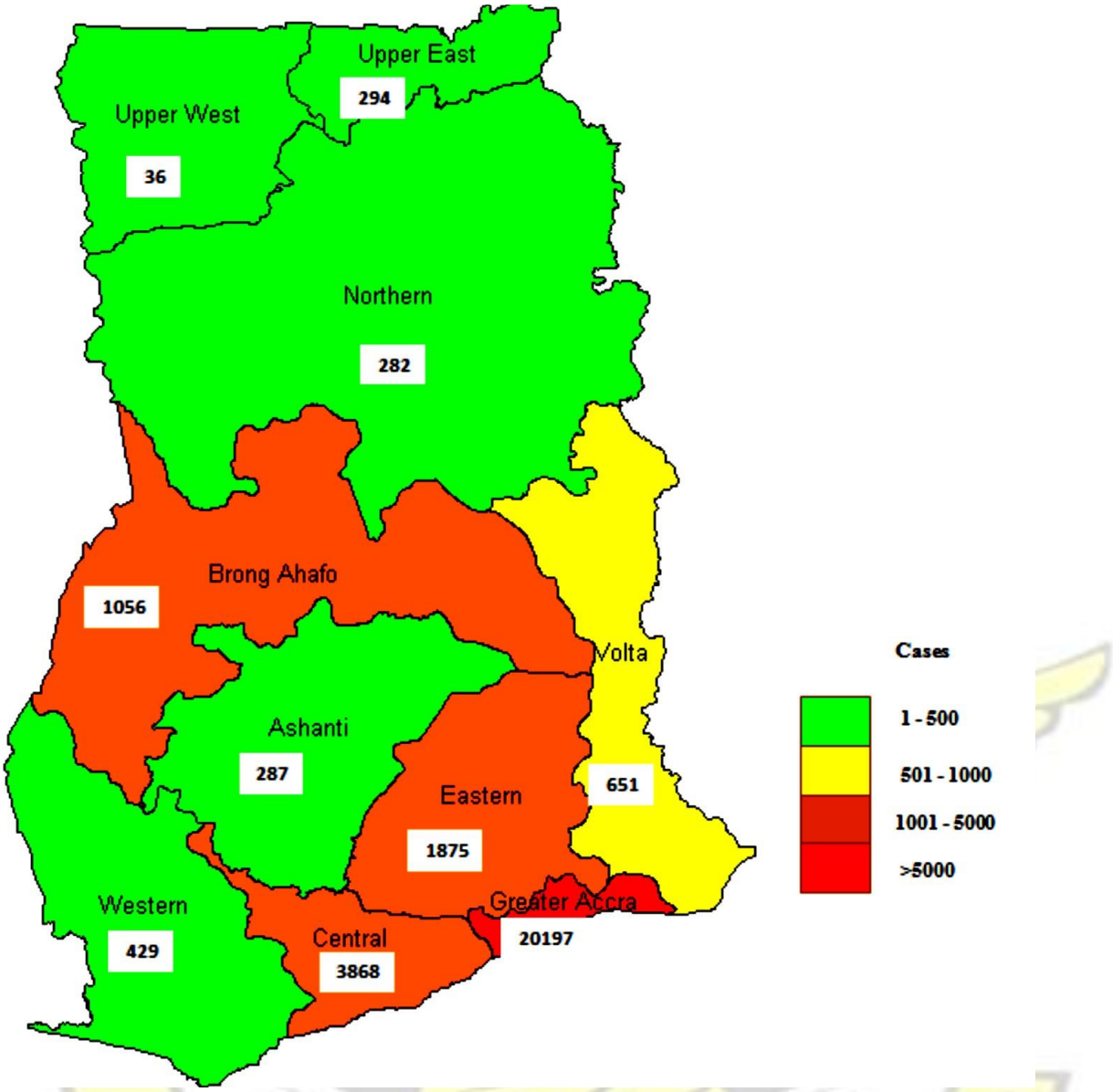
Cholera in Ghana is an urban problem with great effect on the poor who mostly live in overcrowded and filthy areas where the buildings are not arranged in any particular order(Oteng-Ababio, 2014). The extraordinary unregulated population of cities has resulted in deprived environmental conditions such as absence of potable drinking water and the challenge of waste disposal(Oteng-Ababio, 2014).

In June 2014, a cholera outbreak emerged in Ghana, and by 24th August 2014, there were 8,777 cases including 83 deaths (CFR of 0.9%) (WHO, 2014). There were reports from 34 districts in five regions, with Greater Accra being the worst affected region, recording

7,434 cases and 63 deaths (WHO, 2014). By early September, the figure soared to over 14,000, with 126 deaths (WHO, 7th Sep2014). As of 5th October, a collective total of 22,276 cases with 177 deaths was recorded but increased to a total of 24,878 cases including 207 deaths as of 19th October. All the 10 regions of Ghana were affected by the cholera outbreak. Greater Accra was the worst hit site with 75 per cent of cases and 60 per cent of deaths (WHO, 2014). By the end of 2014 (week 52 of the outbreak), 130 out of the 216 districts in all 10 regions recorded a cumulative total of 28,975 cases with 243 deaths (CFR of 0.8%). This is the highest number of cases ever recorded since the beginning of cholera in the country in 1970. Since the start of 2015 until 20th September, a total of 683 cases with four deaths had been reported from 34 districts in nine regions. Greater Accra region, the region with the highest number of cases during the outbreak continued to report cases every week

(WHO, 2015).

Figure 2.1: Distribution of cholera cases in Ghana by region, 2014



(Courtesy of WHO, 2015)

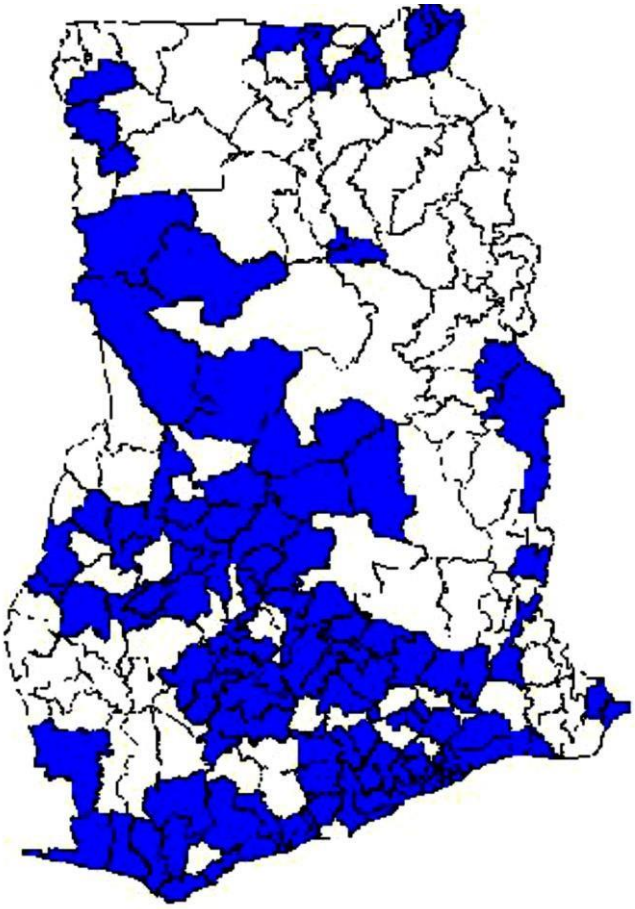


Figure 2.2: Cholera affected districts in Ghana (WHO, 2014)

**** Blue colours indicate cholera affected districts.**

2.5 PATHOGENESIS AND VIRULENCE FACTORS OF CHOLERA

Cholera has a short incubation period of about two hours to five days which increases the explosive nature of the disease during epidemics. Fecal contamination of water or food is the main source of cholera tranmission (Opare et al., 2010). Low pH found in the human stomach allows the organism to be sensitive to it hence an infectious dose of approximately10^8 bacteria per gram is required for the initiation of severe diarrhoea (Kitaoka *et al*., 2011), however, this can be reduced to 10^4 bacteria per gram in people with less stomach acid, such as young children, the aged and those on antacids (Kitaoka *et al*., 2011).The bacteria

moves into the small intestine through the human gastric acid barrier. There, they inhabit, increase and begin to secrete cholera toxin. About 1–5 days after ingesting contaminated food or water, cholera patients experience an abrupt watery diarrhoea and vomiting (Kitaoka *et al*., 2011). A watery diarrhoea up to 20 litres often known as rice-water stool, is released daily containing 10^9 *V. cholerae* per milliliter of stool (Kitaoka *et al*., 2011). In adults, loss of water as a result of cholera may reach one litre per hour, which can lead to severe dehydration, shock and eventually death (Kitaoka *et al*., 2011). Cholera fatality rate in a community can reach 50% within a few hours to days after onset of the disease if left untreated (Kitaoka *et al* .,2011)

2.5.1 *VIBRIO CHOLERAE* TOXINS

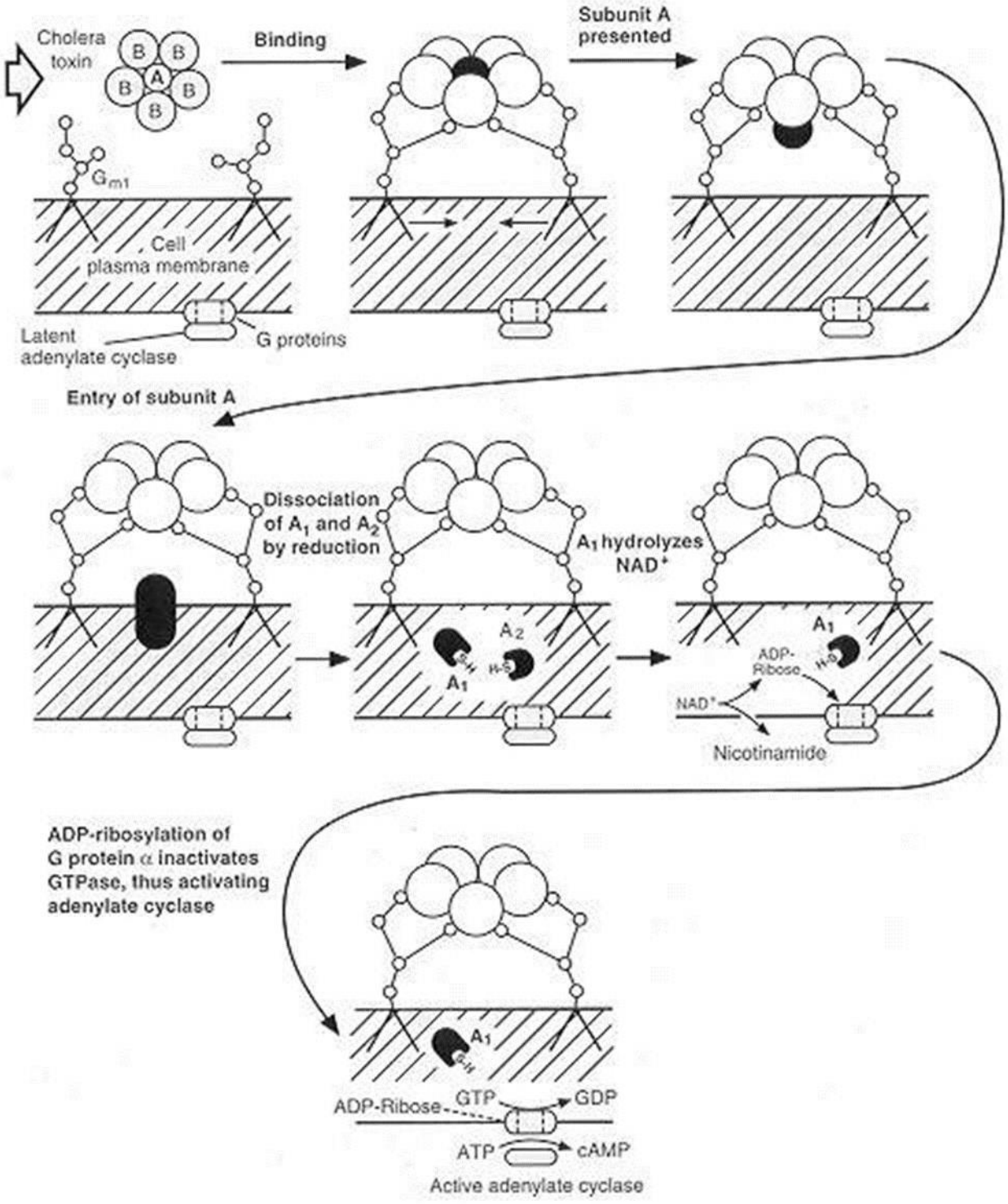
V. cholerae expresses two main virulence factors which are, the Toxin Coregulated Pilus (TCP) and Cholera Toxin.

Cholera toxin (CT) is a major determinant of the virulence of the bacterial *Vibrio cholerae.* The toxin is made up of two A subunit for toxic activity and five B subunit for attachment. Once they attach to the intestinal lumen, A subunit binds with cell membrane receptors (GM1 ganlioside receptor) and induce the activity of the Adenylate cyclase (AC) leading to an increase in cAMP concentration(Sanchez & Holmgren, 2011). High levels of cAMP produce an imbalance in electrolyte mostly sodium and chloride in the epithelial cell by cystic fibrosis trans-membrane conductance regulator(CFTR) which leads to vast fluid loss from the intestine(Sanchez & Holmgren, 2011)

Toxin Coregulated Pilus is encoded in the Vibrio pathogenicity island I (VPI I). It is a type IV pilus that allows the organism to aggregate (Kitaoka et al., 2011). TCP is also required for colonization of the small intestine of infant mice (Taylor et al., 1987) and humans by *V. cholerae*

(Herrington et al., 1988). Out of over 200 serogroups, only serogroup O1 and O139 strains are capable of producing cholera toxin(CT) and toxin coregulated pilus (TCP) which cause epidemics (Paauw et al., 2014). The toxigenicity of a *Vibrio cholerae* strains depends on its ability to produce cholera toxin encoded by CtxAB genes and toxin coregulated pilus encoded by the vibrio pathogenicity island. However nonO1/O139 also exhibits these virulence factors without causing epidemic threat (Paauw et al., 2014). Infrequently, other strains of *V. cholerae* may cause diarrhoea but do not have epidemic potentials (Paauw et al., 2014). For diagnosis of an infectious outbreak, early recognition and identification of the causative microorganism is very crucial. Hence discrimination between epidemic *Vibrio cholerae* O1 and O139 strains and other *Vibrio cholerae* is essential (Paauw et al., 2014).

Fig 2.3: A diagram illustrating the action of cholera toxin



Finkelstein, 1996

2.6 CHOLERA TRANSMISSION

Vibrio cholerae is the bacteria that causes an intestinal disease called Cholera by colonizing the human intestine. Water is the medium by which the disease is transmitted. *Vibrio cholerae* can survive outside the human host in the aquatic environment since it is a natural member of the aquatic microbial community (Bertuzzo et al., 2010). It can spread from the aquatic environment to inland through waterways. The same way it can also spread from the inland regions to the aquatic environment during epidemic outbreaks. Two means of cholera transmission have been described. These are primary and secondary transmissions. Primary transmission occurs from a natural reservoir of *V. cholerae* in the aquatic environment to the human host (Hartley et al.,2006). Secondary transmission occurs through contact with fecally contaminated water sources or food. This mode of transmission is also said to be from human to human (Hartley et al.,2006). Ingestion of contaminated water either by *Vibrio cholerae* present in its natural reservoir (primary route) or contaminated by humans (secondary infection) is always the cause of cholera infection (Bertuzzo et al., 2010).

2.7 SOCIOECONOMIC AND ENVIRONMENTAL RISK FACTORS AFFECTING CHOLERA

Socioeconomic and environmental factors apparently increases the exposure of people to infection and contribute to the spread of cholera epidemic (Mugoya et al., 2008). Socioeconomic and environmental risk factors can also tell the degree to which an infection will reach epidemic extent as well as control the size of the epidemic (Hartley et al., 2006). Studies have shown relatedness of surface water, coastal areas, high population density poverty and low educational status as the major risk factors of cholera in endemic areas (Ali et al., 2002a,

2002b). Lack of basic infrastructure with regards to access to safe drinking water and proper sanitation causes cholera to spreads rapidly in environments (Ali et al., 2002a, 2002b). Outside the human body, the cholera vibrios can live and increase in number. Environmental conditions including improper disposal of liquid and solid waste such as human faeces allows the organism to spread rapidly (Ali et al., 2002a, 2002b). Supply of clean potable water, good sanitary conditions and sewage treatment have long been known as important critical measures for prevention and eradication of the disease cholera. These measures are reported to have eliminated cholera from industrialized and developed countries (Griffith et al., 2006). Chronic poverty and lack of awareness in developing countries makes implementation of these measure almost impossible.

2.8 CHOLERA SYMPTOMS

Cholera symptoms can begin as early as a few hours or as long as five days after infection. Symptoms are often mild depending in part on the size of inoculum. Symptoms such as anorexia, abdominal discomfort, and simple diarrhea are usually the signs of cholera but sometimes the illness may be sudden with profuse watery diarrhoea. A stool with faecal matter is the initial symptoms (Harris et al., 2012), but soon the diarrhoea turns into a pale gray color with an inoffensive but fishy odor (Harris et al., 2012). The typical rice water appearance in the stool is impacted by mucus in the stool. Tenesmus is absent. As large volumes of fluid are being passed effortlessly there is often a feeling of relief. A few hours after the onset of diarrhea is usually vomiting. Severe watery diarrhea accompanied by vomiting is usually seen in about one in 20 people infected with cholera which can quickly lead to dehydration. Although many infected people may have minimal or no symptoms, they can still contribute to the spread of cholera infection (Kaper et al., 1995).

Signs and symptoms of dehydration include:

Rapid heart rate

Loss of skin elasticity

Mucous membranes such as the mouth, throat, nose, and eyelids becoming dry

Low blood pressure

Thirst

Muscle cramps

If not treated, dehydration can lead to hypovelemic shock and death in a matter of hours (Kaper et al., 1995).

2.9 CHOLERA TREATMENT AND PREVENTION

Cholera is a disease of the poor, usually associated with poor hygiene and lack of clean water (Pape & Rouzier, 2014). Provision of adequate sanitation and potable-water system is the most definitive way to prevent and limit the spread of cholera (Pape & Rouzier, 2014). Water contamination from human feces can be reduced by sanitation interventions, such as from latrines to flush toilets. These separates them from drinking water supply there by reducing the rate of contamination (Fung, 2014). Chlorination, boiling or filtering of water reduces the bacterial concentration in drinking water (Fung, 2014). These measures promote access to safe drinking water and reduce "contact" between susceptible populations and contaminated water (Fung, 2014). However, the cost of establishing adequate sanitation systems, is expensive for the countries that are affected by cholera (Pape & Rouzier, 2014).

Cholera vaccination is another option for cholera prevention and control (WHO, 2010). In countries endemic with cholera, use of cholera vaccines are gradually being accepted as a useful supplement to improving water, sanitation, and hygiene (WHO, 2010).

Oral or intravenous hydration is the main stay of cholera treatment. Treatment with antibiotics is recommended in conjunction with hydration for severely ill patients. Doxycycline is the recommended first-line treatment for adults where as azithromycin is the recommended first-line treatment for children and pregnant women. Orfloxacin, trimethoprim/ sulfamethoxazole, chloramphenicol and ciprofloxacin are all effective to cholera but tetracycline is more effective than them (CDC, 2015).

2.10 CHOLERA VACCINES AND IMMUNITY

Two oral cholera vaccines have been approved for use by WHO. They are Dukoral (Crucell, Stockholm, Sweden) and Shanchol (Shantha Biotechnics, Hyderabad, India). Dukoral and Shanchol are whole cell, killed vaccines. Both vaccines require two doses administered about 7-14 days apart. Dukoral requires a buffer of 75-150mls of clean water while Shanchol does not require a buffer(Date et al., 2011). A third cholera vaccines named mORCVAX is manufactured solely in Vietnam (Pape &Rouzier, 2014). There is proof of safety and effectiveness (66 to 85%) after two doses, with immunity lasting up to 5 years for all three vaccines (Pape & Rouzier, 2014). WHO recommendations for the use of oral cholera vaccines are

1. OCV should not replace usually recommended control measures such as adequate sanitation, improved water supplies and health education but should always be used

as an additional public health tool which needs to be linked to strengthen surveillance and early warning.

2. Due to its 2-dose regimen and the time required to reach protective efficacy, high cost and heavy logistics associated with its use, the cholera vaccine is not be administered once a cholera outbreak has started (WHO,2016)

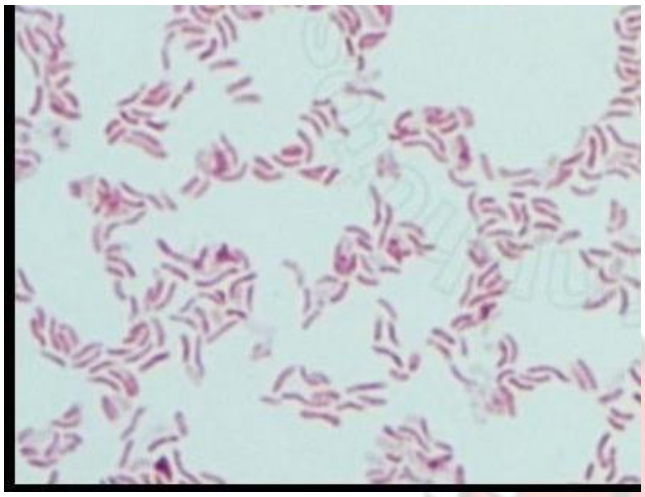
Again they are not routinely used in cholera outbreaks because of limited availability of vaccines, complex challenge of multidose regimen(Date et al., 2011).

2.11 STRUCTURE AND CLASSIFICATION OF *VIBRIO CHOLERAE*

The family *Vibrionaceae* comprises of *Vibrio* spp, *Aeromonas*, *Phobacterium*, and *Plesiomonas* spp (Kaper et al., 1995). *V. cholerae* are gram-negative rods, motile by a single polar sheathed flagellum, facultatively anaerobic, asporogenous bacteria. *V. cholerae* is oxidase positive, reduces nitrate to nitrite (Kaper et al., 1995). Isolates require an inorganic nitrogen source, utilizable carbohydrate, and appropriate minerals to grow well. Rapid growth with a generation time of less than 30 minutes is often seen when media is adequate. Vibrios are sensitive to low pH and die rapidly in solutions below pH 6. They however have the ability to survive under alkaline conditions (Finkelstein, 1996).

Before 1992, there were two biotypes of *Vibrio cholerae* that cause epidemic cholera. Namely, Classical and El Tor. Koch was the first to isolate Classical *Vibrio cholerae* in the year 1883. Later, other vibrios similar to *Vibrio cholerae* were isolated from Mecca-bound pilgrims at the quarantine station at El Tor, in the Sinai Peninsula in the early 1900s (Finkelstein, 1996). S

Fig 2.4: Gram stain appearance of *Vibrio cholerae* under the microscope



Courtesy: www.Bacteriainphotos.com

2.11.1 SEROTYPE

It refers to the different antigenic forms of the O1 antigen. The O antigen is the main surface antigen used in characterization of *V. cholerae*. It is heat stable and is composed of a homopolymer containing amino acid sugar. D-perosamine in which the amino acid group are acylated by 3-deoxy-L-glycero-tetronic (Kaper et al., 1995).

2.11.2 SEROGROUP

The term serogroups or serovars refers to the different O groups. Serogroup O1 and O139 are the only serogroups known to cause epidemic and pandemic cholera out of over 200 serogroups (Marin et al., 2013; Okada et al., 2012). The presence of a common flagella (H) antigen among all *Vibrio* species makes its use in species identification limited (Mitra et al., 2001).

2.11.3 *VIBRIO CHOLERAE* O1

The O1 serogroup of *V. cholerae* that produces CT has been long ago associated with epidemic and pandemic cholera (Kaper et al., 1995). There are two biotypes of serogroup O1. These are Classical (CL) and El Tor (ET) (Chomvarin et al., 2013). The O1 serogroup has three serotypes namely, Ogawa, Inaba and Hikojima (Okada et al., 2012)

2.11.4 *VIBRIO CHOLERAE* O139

In late 1992, strains of *V. cholerae* arose as the cause of epidemic cholera but did not agglutinate with the O specific antisera. This was later designated as O139. Bengal in recognition of the origin of the strain (Kaper et al., 1995). *Vibrio cholerae* O139 strains possess a virulence factors common to O1 El-Tor and causes diarrhoea which is clinically indistinguishable from O1 cholera (Kaper et al., 1995). Unlike *Vibrio cholerae* O1, O139 possess a short truncated form of Lipopolysaccharide (LPS) and produce a polysaccharide capsule which mediates adherence to epithelial cells (Mitra et al., 2001).

2.11.5 *VIBRIO CHOLERAE* NON O1/ NON O139

Non-O1 *V. cholerae* refers to all *Vibrio cholerae* isolates before the appearance of the O139 serogroup classified *as V. cholerae* on the basis of biochemical tests but negative for the O1 serogroup (Kaper et al., 1995). These strains are usually found in estuarine environments and do not produce CT hence not linked with epidemic diarrhoea but occasionally, they are isolated from diarrhea cases (Kaperet al., 1995).

2.11.6 BIOTYPING

Over the years, strains of *V. cholerae* O1 have been grouped into two biotypes, namely classical and El-Tor (Kaper *et al.*, 1995). The classical biotype was found to have caused the first to the sixth pandemic where as isolates from the seventh pandemic were of the El-Tor biotype.

Table 2.1 Differentiation of classical and El-Tor biotype of *V. cholerae*

Test	**Results for biotype classical**	**El-Tor**
Hemolysis	–	+
Agglutination of chicken erythrocytes	–	+
Voges-Proskauer	–	+
Inhibition by polymyxin B (50-U disk)	+	–
Lysis by		
Classical IV bacteriophage	+	–
FK bacteriophage	+	–

(Kaper et al., 1995).

2.12 DIAGNOSIS AND IDENTIFICATION OF VIBRIO

Isolation of *Vibrio cholerae* especially O1 is extremely essential for cholera outbreak confirmation. Rapid diagnostic tests made to detect lipopolysaccharide in fecal specimen can be employed to assist in early detection as well as surveillance (Keddy et al., 2013). In

addition, other tests are available for the identification of the bacteria and these include the serogroups, serotypes and biotypes. In order to achieve isolation of *V. cholera*, experts often recommended using Cary-Blair transport medium for fecal cholera specimen (Keddy et al., 2013). The fecal specimen is then subcultured onto both a nonselective and a selective media such blood agar and TCBS (Thiosulphate Citrate Bile Salt Sucrose) agar respectively. After a period of incubation, usually between 18 to 24 hours at 37°C, isolates which show oxidase-positive are further serotyped using a polyvalent and a monovalent antisera (Keddy et al., 2013). Having isolated the bacteria, it then becomes expedient to determine the resistance of the bacterial to antibiotics using any of the standard Antimicrobialsusceptibility methods. With modern advancement in science, molecular characterization of *V. cholera* has become essential in phenotypic identification and also in the investigations of outbreaks. Molecular characterization using Polymerase chain reaction (PCR) can confirm if genes that encode lipopolysaccharide and cholera toxin are present and as well distinguish between El Tor biotype traits (Keddy et al., 2013).

2.12.1 SPECIMEN COLLECTION

Stool samples are the ideal samples from which *V. cholerae* are isolated. Thus during outbreaks, stools are collected from participants using sterile rectal swabs and placed in Cary-Blair transport medium if processing is likely to be delayed. However, it is recommended that samples be taken preferably before the start of an antibiotic therapy. In the absence of Cary-Blair, tellurite-taurocholate-peptone broth as well as alkaline peptone water could serve as enrichment medium for *V. cholera*. However, care should be taken not to prolong holding of

specimen in Alkaline peptone water as this can promote growth of non- O1 *V. cholera* as well as *Pseudomonas* spp more especially at ambient temperatures

(Kaper et al., 1995).

2.12.2 BACTERIA CULTURE

Thiosulfate-citrate-bile salts-sucrose (TCBS) agar remains the widely used medium for the plating of *V. cholerae.* This is because, upon sucrose fermentation of *V. cholerae*, the colonies appear on the medium as large smooth yellow colonies and can be confirmed by oxidase test. However, when oxidase test is performed on colonies directly obtained from growth on TCBS, there is a good chance the test may give a false result. It is therefore recommended that colonies from the TCBS should be subcultured onto a nonselective medium, preferably blood agar before testing for oxidase and agglutination with both polyvalent and monovalent antisera. (Tamrakar et al., 2006).

Fig 2.5: *Vibrio cholerae* appearance on TCBS agar

Courtesy: www.bacteriaphotos.com

2.12.3 DIAGNOSIS OF *VIBRIO CHOLERAE* USING PCR

PCR provides a better alternative to microscopy, culture as well as biochemical tests for the identification of *V. cholerae* and its speciation (Singh & Mohapatra, 2008). Several PCR protocols are available for characterization of serogroups, biotypes, and the toxigenic potential of *Vibrio cholerae* strains (De et al., 2001). It should be noted that conventional PCR methods are not recommended for routine use. This is because the gel electrophoresis used for analyzing the results have a high risk of contamination and is labour intensive (De et al., 2001). Real time PCR on the other hand, makes detection of accumulated products of amplification possible through the changes in the fluorescence intensity within a closed tube. Thus making real time PCR move sensitive and faster when compared to conventional PCR. Real time PCR has therefore gained popularity in clinical microbiology labs. The usage of probes labelled with multicolour fluorophore together with or without melting curve analysis gives multiplex real time PCR an added advantage of detecting several target genes simultaneously in a single reaction tube (Fukushima et al., 2003). By far, a lot of published works on *V. cholerae* that used real time PCR assays could not detect more than two genes simultaneously (Fukushima et al., 2003) thus limiting its use for simultaneous toxin status and serotype determination. As mentioned above, using different fluorophore labeled probes with or without melting curve analysis greatly improves both sensitivity and specificity of multiplex real-time PCR in the detection of *V. cholerae* (Gubala & Proll, 2006).

2.13 ANTIMICROBIAL SUSCEPTIBILITY OF *VIBRIO CHOLERAE*

Previous studies show that *V. cholerae* isolates are El-tor biotype, serotype Inaba or Ogawa (Bhat et al., 2012). Treatment with antibiotics shortens the duration of illness in cholera and also reduces the period during which vibrios are excreted (Bhat et al., 2012). However, in cholera disease management and control, resistance to antibiotics poses a significant medical and public health challenge (Faruque et al., 2006). Although antibiotics such as streptomycin, norfloxacin, ciprofloxacin, tetracycline and doxycycline are often administered as adjuncts during rehydration therapy, resistance in some species of *Vibrio* including *V. parahaemolyticus, V. cholerae, V. vulnificus* and *V. fluvialis* have been recordeds (Chiang & Chuang, 2003; Lima, 2001).

A study by (Mukhopadhyay et al., 1996) showed an increase in the frequency of strains of *V. cholerae* that are drug resistant. A more serious clinical problem is created upon the emergence of microbial strains that show multiple drug resistance, thus in cholera-like diarrhea, treatment and containment becomes more difficult. This can further be illustrated in the light of the cholera epidemic that occurred in Guinea-Bissau from 1996 to 1997 (Dalsgaard et al., 2001). The emergence of strains that were resistant corresponded with the increase from 1% to 5.3% in fatality rate. Bhat et al in 2012 reported resistance to Cotrimoxazole, doxycycline and ampicillin but sensitive to ciprofloxacin, ofloxacin, cephotaxime and azithromycin (Bhat et al., 2012). SXT element is a genetic element which bears similar properties as conjugative transposons and carries genes that encode resistance to streptomycin, trimethoprim and sulfamethoxazole in *V. cholerae* strains (O139 and O1) that were isolated in India and yet was absent in O1 strain isolated in 1994 from refugees from Rwandan in Goma, Zaire (Waldor & Tscha, 1996). Earlier reports had shown that gene cassettes within

class1 integrons were associated with various *V. cholerae* O-serotypes that are mostly clinically originating from Thailand (Dalsgaard et al., 2000). Another study in South Africa on SXT elements as well as class 1 integrons, revealed the presence of these elements in clinical isolates from Africa (Dalsgaard et al., 2001). Other studies by (Opintan et al., 2008) on isolates of *V. cholerae* O1 Ogawa outbreak, detected cassettes that are as similar as Tn7 in the class 2 integrons. This transposon although often in enteric organisms are more so found either integrated into the chromosome at specific attachment sites or on conjugative plasmids. Here in Ghana, Ghana Health Service recommends erythromycin, chloramphenicol, tetracycline, azithromycin and doxycycline as treatment for cholera (Kuma et al., 2014).

Unfortunately, chloramphenicol and erythromycin have recorded high resistance levels (66.7% and 94.4% respectively), thus rendering them unsafe in managing cholera in Ghana (Kuma et al., 2014). However, all isolates studied by kuma et al in 2014 were sensitive to azithromycin and showed low resistance to ciprofloxacin (0.4%), doxycycline (14.5%) and tetracycline (15.6%)(Kuma et al., 2014). Still in Ghana, another pilot study of 27 *V. cholerae* isolates from the capital city Accra in 2006, also revealed resistance to conventional antibiotics including trimethoprim and nalidixic acid used in treatment. On the other hand all isolates were susceptible to levofloxacin with 24 of the isolates being susceptible to tetracycline (Opintan *et al*., 2008). According to Kuma *et al* 2014, cholera isolates from the Atebubu-Amanten district outbreak recorded between March to July 2012 in the Brong Ahafo region, Ghana, also exhibited intermediate susceptibilities and resistance to all the antibiotics used in the study including nalidixic acid, chloramphenicol, cotrimoxazole: sulfamethoxazole/trimethoprime, erythromycin, ampicillin and tetracycline except ciprofloxacin.

This study focuses on determining the molecular characteristics and antimicrobial susceptibility profile of *Vibrio cholerae* isolates obtained in the 2014 outbreak in Ghana. This included isolation, serotyping, antimicrobial susceptibility pattern, detection of virulence genes and fingerprinting of the isolates by PFGE.

CHAPTER THREE

METHODOLOGY

3.1 STUDY DESIGN

This was a retrospective study in which stored rectal swabs which were positive for *V. cholerae* were obtained and cultured. They were obtained from previously cultured samples at the National Public Health Reference Laboratory (NPHRL), Korle-Bu, Hospital and Komfo-Anokye Teaching Hospital. The swabs were cultured for *Vibrio cholerae*. The isolates were serotyped and tested for their antibiotic susceptibilities. This was done over a two month period (March-April, 2015), after which the presence of virulence genes and SXT element were determined using PCR.

3.2 ETHICAL CONSIDERATION

Ethical approval was obtained from the Research and Ethics Committee of the Kwame Nkrumah University of Science and Technology. Authorization to collect samples was also obtained from the authorities of National Public Health and Reference Laboratory, KorleBu, Accra.

3.3 STUDY SITE

The study sites were National public health and reference laboratory (NPHRL) Korle-bu, Accra. Komfo-Anokye Teaching Hospital, Kumasi. The NPHRL is a health institution under the Ghana Health Service, which surveys all public health diseases under investigation, and coordinates laboratory results across Ghana.

3.4 SAMPLE SIZE

The sample size was determined by using a single population formula considering the following assumptions. Za/2=1.96 for the standard scale of 90% level of confidence, level of precision of 5% and prevalence, P of 50% (0.5) (Raosoft Inc., 2004).

Given the formula $N= [Z^2 (P) (1-P)] / (Error)^2$

Where N= Sample size, Z= 1.96, Error = 5%, P= 50%

A sample size of **271** was calculated

3.4.1 Limitations

A total of 62 samples were obtained. The calculated sample size was not obtained because when we tried getting samples from the various public health laboratories across the country, we were not successful

3.5 LABORATORY TECHNIQUES

3.5.1 Sample collection

The NPHRL receives samples (rectal swab) of cholera suspected cases from several health facilities within the Greater Accra region (GAR) and Central Region. Usually, after a *Vibrio cholerae* bacterium has been isolated, positive rectal swabs were kept in a 2ml cryo-tubes containing Tryptose soy broth with glycerol and labelled appropriately to be stored in a freezer at -80^{o}C. These stored positive rectal swabs together with newly diagnosed cholera cases were cultured to obtain *Vibrio cholerae* isolates from NPHRL while one (1) isolate was obtained from KATH.

3.5.2 Isolation of *Vibrio cholerae* from rectal swab

Upon receiving suspected *Vibrio cholerae* samples (rectal swab) at the NPHRL, samples were cultured on TCBS agar (day one) after which the samples were inoculated into alkaline peptone water. The agar plate and the alkaline peptone water were both incubated overnight. After the overnight incubation, the TCBS plates were inspected for vibrio colonies. If no vibrio colonies grew, the swab from the alkaline peptone water was subcultured on TCBS agar (day two) and incubated overnight. The cultured plates were inspected for vibrio colonies. TCBS plates without growth were discarded with their sample (day three). Colonies appearing yellow on TCBS suspected to be vibrio were picked clearly and inoculated onto Tryptose soy agar medium and incubated overnight at 37^{o}C to obtain a pure culture.

3.5.2 Biochemical and Serological Testing

Distinct colonies were picked and tested against oxidase reagent for the characteristic purple colour within a few seconds. Colonies which were oxidase positive were then serotyped first with polyvalent antisera and then monovalent anti-sera (DENKA SEIKEN CO., LTD. TOKYO, JAPAN) for *V. cholerae* O1 or O139. A drop of normal saline was kept on a glass slide. A colony of *vibrio cholerae* was picked from the agar plate and emulsified in the drop of the normal saline after which a drop of the polyvalent anti-sera was added and mixed well for the presence of agglutination. Samples that showed positive (agglutinated) with the polyvalent anti-sera were further tested with the monovalent antisera to determine if they were Ogawa, Inaba or Hicojima.

3.5.4 Antibiotic susceptibility testing

Using the Kirby-Bauer disc diffusion method on Mueller– Hinton (MH) agar, the antimicrobial susceptibility patterns of the *V. cholerae* isolates were determined. In doing this, pure colonies from Tryptose soy agar were emulsified in 2ml of sterile peptone water to obtain a turbidity comparable to the standard 0.5 McFarland. A swab stick which is sterile was immersed into the inoculum tube and pressed against the side of the bottle above the inoculum to get rid of extra fluid from it. The surface of the MH agar plates were allowed to dry before being streaked with the swap stick. The entire agar surface was streaked and this was done by turning the agar plates approximately 90 degree each time to ensure the inoculum is evenly distributed. With the help of forceps, the appropriate antibiotic discs were placed on the agar and incubated at 37^{o}C overnight (18-24hrs).

Using a venier caliper, the diameter of zones of inhibition were measured and the sizes compared to a standard obtained from the NPHRL to determine susceptibility or resistance. The antibiotic discs tested and their concentrations were Tetracycline 30ug, Doxycycline 30ug, Ciprofloxacin 5ug, Erythromycin 15ug, Cefuroxime 30ug, Ceftriaxone 30ug, and Chloramphenicol 30ug Cotimozaxole 25ug, Ampicillin 10ug.

3.6 DETECTION OF GENES ENCODING *VIBRIO CHOLERAE* VIRULENCE AND SXT ELEMENT

3.6.1 DNA Extraction

Vibrio cholera DNA was extracted using Spherolyse extraction kit obtained from HAIN LIFESCIENCE. An overnight culture was prepared in microcentrifuge tubes containing

Brain Heart Infusion Broth. The tubes were span in a centrifuge to get the isolates to the bottom whiles the BHI remains at the top. The supernatant was then decanted using a pipette whiles the isolates were left at the bottom of the tube. These were then thoroughly eluted into the spherolyse tube for a period of 10seconds. The S-lyse tube containing the *vibrio cholerae* isolates were closed and votexed at maximum speed of 14,000rpm for 10minutes, after which the S-lyse tubes were incubated at 95^{o}C for 15minutes in a thermo mixer after which it was span in a centrifuge for 2minutes at full speed(14000RPM). The supernatant containing the extracted DNA were then pipetted into a 1.5ul eppendorf tubes and stored at 20^{o}C.

3.6.2 Aliquoting of Primer

Lyophilized primer pairs obtained from INTERGRATED DNA TECHNOLOGIES for cholera toxin, toxin coregulated pilus and SXT elements. The primer pairs were adopted from Menezes et al, 2014 and Ramachandra et al, 2007 as shown in table 3.1 below

Table 3.1: Primer sequence used in the study and genes they target

Genes	Primer sequence	Source
ctxAB(cholera toxin)	F:5'-gcc gggttgtgggaatgctccaag 3' R:5'-gcc atactaattgcggcaatcgcatg 3'	Menezes et al, 2014
tcp(toxin co-regulated pilus)	F:5'- cgttggcggtcagtcttg- 3' R:5'- cgggctttcttcttgttc g - 3'	Menezes et al, 2014
SXT	F:5'- atggcgttatcagttagctggc-3' R:5'-gcgaagatcatgcatagacc-3'	Ramachandranet al, 2007

3.7 DNA AMPLIFICATION

Table 3.2: Pipetting scheme for PCR amplification of SXT master mix

Reagents	Per sample(ul)
Nuclease free water	35.75
Magnesium chloride	4
X10 buffer	5
Firepool Tag polymerase	0.25
SXT forward primer	1
SXT Reverse primer	1
dNTPS	1
DNA template	2
Total reaction volume	**50 ul**

3.7.1 a, Cycling conditions for cholera toxin (CtxAB)

Initial denaturation; 94^{o}C for 3minutes, Denaturation; 94^{o}C for 1 minute, Annealing; 78^{o}C for 30 seconds, Extension; 72^{o}C for 1 minute, Final extension 72^{o}C for 10 minutes. A total of 34 cycles was obtained.

b, Cycling conditions for Toxin Coregulated Pilus (TCP)

Initial denaturation; 94^{o}C for 3 minutes, Denaturation; 94^{o}C for 1minute, Annealing;59^{o}C for 30 seconds, Extension;72 ^{o}Cfor 1 minute; Final extension 72^{o}C for 10 minutes

c, Cycling conditions for SXT gene

Initial denaturation; 94^{o}C for 3 minutes, Denaturation; 94^{o}C for 1 minute, Annealing; 62^{o}C for 30 seconds, Extension; 72^{o}C for 1 minute, Final extension; 72^{o}C for 10 minutes

3.7.2 Preparation of Gel Electrophoresis

A 1.5% Agarose gel was prepared from 40mls of 0.5 TBE buffer. To do this, 0.6grams of agarose was weighed and added to 40mls of 0.5 TBE buffer. The solution was microwaved for 2 minutes for the agarose to dissolve completely. 2ul of ethidium bromide was added and mixed by swirling. The mixture was then poured into a gel tray with a comb and left to solidify. The combs were taken off and the gel was repositioned in the tank. 2ul of loading dye was mixed with 10ul of pcr template as well as positive and negative control and pipetted into the wells created by the comb. The first well was loaded with peglab 100bp ladder, the second with the positive control and the third with the negative control followed by the samples. The gel tank was closed and the power button switched on. The gel was run at 100 volts for 45minutes to obtain well separated bands. The gel was removed and viewed under UV light for the presence of bands after the 45 minutes.

CHAPTER FOUR

RESULTS

A total of 62 samples were obtained, of which one was from Komfo-Anokye Teaching Hospital whiles 61 were from NPHRL-Korle Bu. Out of the 62, 40 *Vibrio cholerae* isolates were recovered. One isolate was obtained from Komfo-Anokye Teaching Hospital, whiles the

rest 39 were obtained from NPHRL, Korle-Bu. The rate of isolation for KATH was 100% and that of NPHRL was 64% and the overall rate of isolation was 65%.

Table 4.1: Distribution of *Vibrio cholerae* isolates in relation to location of isolation

Location	Number of isolates	(%)
KATH	1	1.6
NPHRL	39	98.4

Toxin coregulated pilus (TCP) and cholera toxin (CT) genes were detected in 39 out of the forty *Vibrio cholerae* isolates representing a percentage of 97.5% of the isolates tested. One isolate representing 2.5% did not possess the TCP and CT genes.

Table 4.2: Distribution of CtxAB and TCP genes among the *Vibrio cholerae* isolates

		Frequency	Percent	Total
CTxAB	positive	39	97.5	40
	negative	1	2.5	
TCP	positive	39	97.5	40
	negative	1	2.5	

In table 4.3, 39(97.5%) of the *Vibrio cholerae* isolates were positive for both *vibrio cholerae* toxin gene and toxin coregulated pilus gene whiles one (2.5%) isolate out of the 40 was negative for both CtxAB and TCP gene. There was no isolate that was positive for TCP gene and negative for CT gene.

Table 4.3: Cross tabulation between CtxAB and TCP

	CTxAB		Total
	Positive	Negative	

TCP	Positive	39	0	39
	Negative	0	1	1
Total		39	1	40

40 *Vibrio cholera* isolates were tested against various antibiotics. *Vibrio cholerae* isolates were 95% resistant to Cotrimoxazole (T/S), 90% resistance to Ampicillin and 75% resistance to Ceftriaxone but 2.5% resistant to Cefuroxime and Doxycycline. No isolate was resistant to Ciprofloxacin, Chloramphenicol and Tetracycline

Table 4.4: Frequency distribution of antibiotics resistance profile

Antibiotic name	Number	%R	%I	%S
Ampicillin	40	90	5	5
Cefuroxime	40	0	17.5	82.5
Ceftriaxone	40	75	12.5	12.5
Ciprofloxacin	40	0	0	100
Trimethoprim/Sulfamethoxazole	40	95	0	5
Erythromycin	40	2.5	95	2.5
Chloramphenicol	40	0	35	65
Doxycycline	40	2.5	0	97.5
Tetracycline	40	0	0	100

The Figure 4.1 represents the susceptibility profile of the various antibiotics on *Vibrio cholerae* isolates. All 40 isolates (100%) were susceptible to Ciprofloxacin and Tetracycline whiles 39 (97.5%) of the isolates were susceptible to Doxycycline. Isolates were 82.5% susceptible to Cefuroxime but less than 20% susceptible to Ceftriaxone, Ampicillin, Erythromycin and Trimethoprim/Sulfamethoxazole.

120

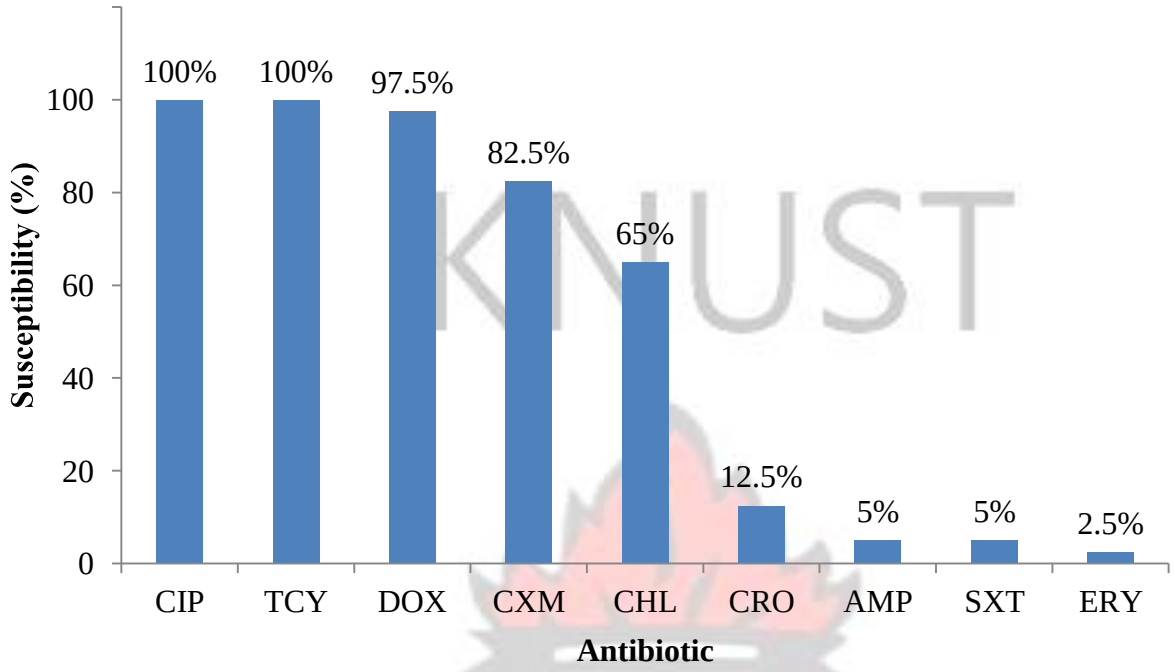


Figure 4.1: Antimicrobial susceptibility pattern of *Vibrio cholerae* isolates of the 2014 outbreak in Ghana

Key**;** CIP=Ciprofloxacin, TCY= Tetracycline, DOX= Doxycycline, CXM = Cefuroxime, CHL= Chloramphenicol, CRO = Ceftriaxone, SXT = trimethoprim/sulfamethoxazole, AMP = Ampicillin, ERY = Erythromycin

The results on *Vibrio cholerae* isolates resistance pattern to antibiotics are presented in Table 4.5. 19 (47.5%) *Vibrio cholerae* isolates cultured from NPHRL were resistance to Ampicillin, Trimethoprim/ sulfamethoxazole, and ceftriaxone. Eight (20%) of the isolates obtained from NPHRL were resistance to ampicillin, chloramphenicol, trimethoprim/sulfamathoxazole and ceftriaxone. The only isolate obtained from KATH was resistance to trimethoprim/ sulfamethoxazole, but sensitive to other antimicrobials tested.

Also, one (2.5%) isolate obtained from NPHRL was resistance to only ceftriaxone but sensitive to all other antimicrobials tested as presented in Table 4.5

Table 4.5: *Vibrio cholerae* isolates resistance pattern to antibiotics

Antibiotics	Number of isolates resistant	% of Isolates	Location at which isolate was obtained
CRO	1	2.5	NPHRL
SXT	1	2.5	KATH
AMP	1	2.5	NPHRL
AMP SXT CRO	19	47.5	NPHRL
AMP CHL SXT	3	7.5	NPHRL
AMP SXT CXM CRO	3	7.5	NPHRL
AMP CHL SXT CRO	8	20	NPHRL
AMP SXT DOX CXM CRO	1	2.5	NPHRL
AMP CHL SXT CXM CRO	3	7.5	NPHRL

In table 4.6, 38(100%) *vibrio cholerae* isolates showed resistance to cotrimoxazole and also indicated the presence of SXT resistant element by PCR. One (50%) isolate was susceptible to cotrimoxazole but showed the presence of SXT element while one (50%) isolate was also susceptible to cotrimoxazole but negative to SXT element. The study showed 14(100%) intermediate resistance to chloramphenicol as well as presence of SXT element. Also, 24 (92.3%) isolates showed the presence of SXT element but susceptible to chloramphenicol. Two (5.0%) isolates were negative for SXT element and also susceptible to chloramphenicol.

Table 4.6: A cross tabulation table between antibiotic susceptibility and SXT element

of PCR

Antibiotic		**SXT element**		**Total**
		Positive	**Negative**	
Cotrimoxazole	**Resistant**	38 (100%)	0 (0.0%)	**38**
	Susceptible	1 (50.0%)	1 (50%)	**2**
Total		**39 (97.5%)**	**1 (2.5%)**	**40**
Chloramphenicol	**Intermediate**	14 (100%)	0 (0.0%)	**14**
	Susceptible	24 (92.3%)	2 (7.7%)	**26**
Total		**38 (95.0%)**	**2 (5.0%)**	**40**

The f

ig: 4.2 illustrates the presence of cholera toxin gene (CtxAB) in *Vibrio cholerae* isolates. The cholera toxin gene was found in 39 out of the 40 isolates. L= Ladder, +C = Positive control, -C= Negative control, lanes 4,5,6,7, and 8 were test lanes for CtxAB (Cholera toxin). Ladder size = 536bp

Figure 4.2: PCR results showing CtxAB (cholera toxin) gene.

The f

ig 4.3 illustrates *Vibrio cholerae* showing the presence of toxin coregulated pillus gene (TCP). This gene was present in 39 out of the 40 *Vibrio cholerae* isolates. L= Ladder, +C= Positive control, -C = Negative control, Lanes 26,28,32,33, and 35 were test lanes for TCP (Toxin coregulated pilus). Ladder size = 805bp

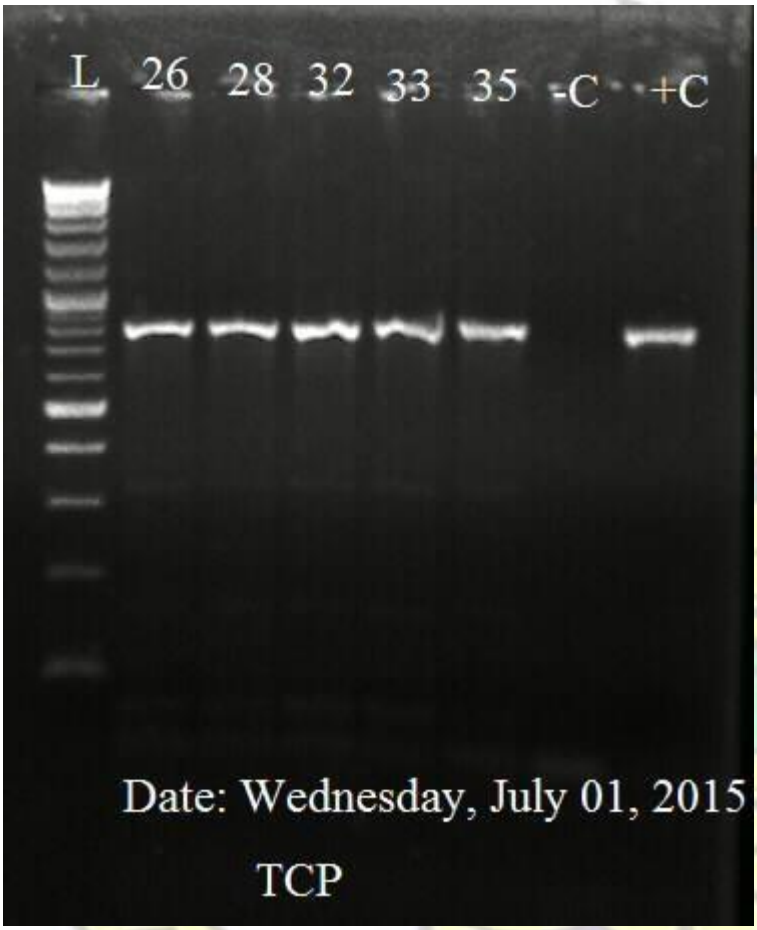


Figure 4.3: PCR results showing TCP (toxin coregulated pillus) gene

The f

ig 4.4 illustrates the presence of SXT element in *Vibrio cholerae* isolates. The SXT element was present in 39 of the isolates. L= Ladder, +C= Positive control, -C= Negative control, Lanes 26,27,28,29 and 30 were test lanes for SXT element. Ladder size= 1035bp

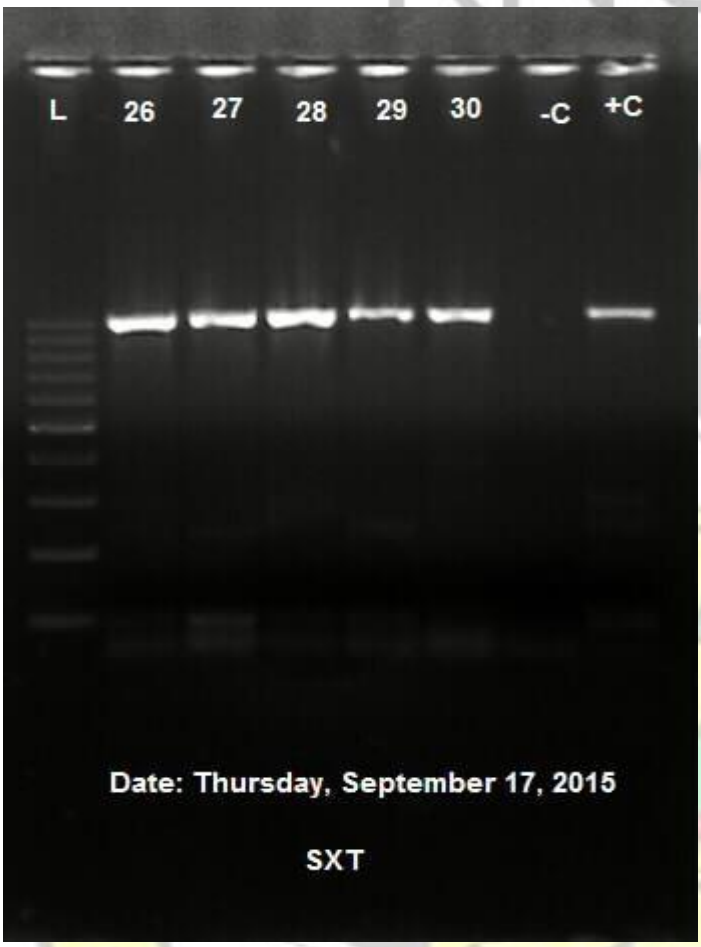


Fig 4.4: A PCR results showing the presence of SXT element in *Vibrio cholerae* isolates obtained in Ghana.

The f

Eight of the isolates produced a good pattern on the PFGE as illustrated in fig 4.5 which was then captured into a Bionumeric software for dendrogram analysis. The isolate on lane 8 gave problems (strange pattern) with PFGE. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 are test strains

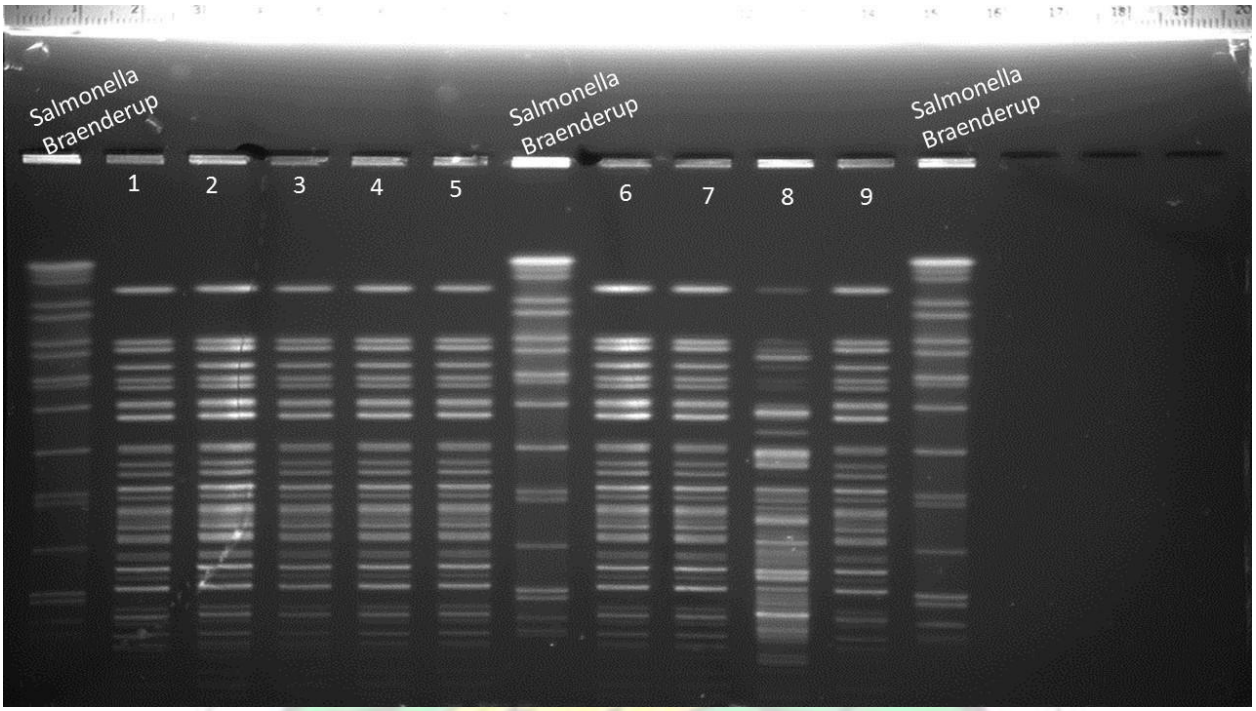


Figure 4.5: PFGE on *V. cholerae* O1

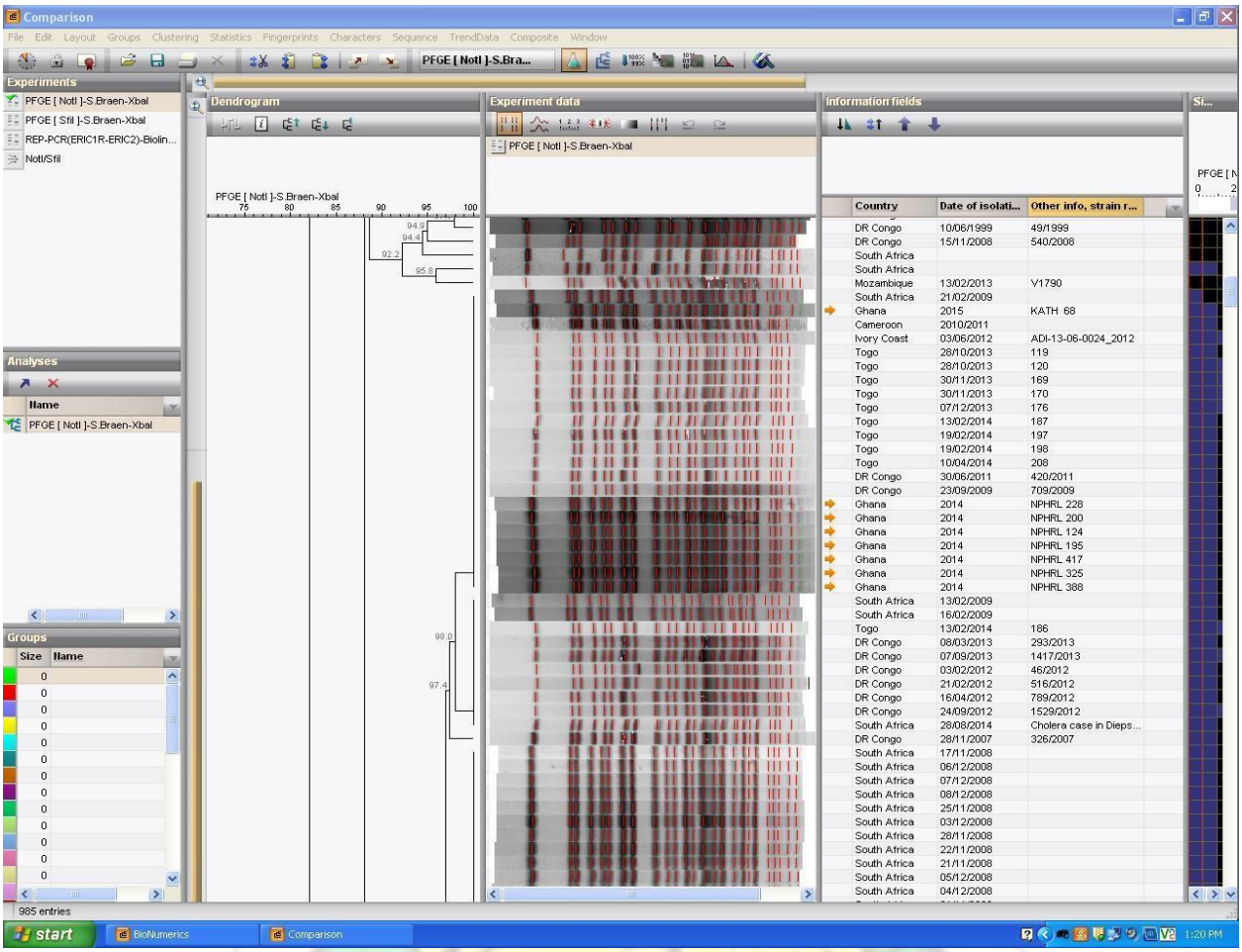


Fig 4.6 Dendrogram analysis of *Vibrio cholerae* isolates from Ghana.

Eight (8) isolates produced a good pattern which were captured into BioNumeric software for dendrogram analysis. The isolates cluster together with the same pattern and share a pattern previously seen in isolates from DRC, Togo, Cameroon and Ivory Coast

4.2 DISCUSSION

Sixty one (61) rectal swab from NPHRL-Korle-Bu were cultured for *Vibrio cholerae*. Thirty-nine (39) of the rectal swabs were positive for vibrio. An additional isolate from

KATH made a total of forty (40) isolates. The isolates were characterized as *Vibrio cholerae* O1 serotype Ogawa a serotype associated with cholera outbreak in Africa (Opinta et al., 2008).

PCR analysis of the isolate gave 39(97.5%) of the isolates to be positive for the cholera toxin gene (CT) and toxin coregulated pillus (TCP). These genes are both needed by *Vibrio cholerae* for its pathogenesis.

All forty *Vibrio cholerae* isolates were tested for antimicrobial susceptibility against antimicrobial agents, namely ciprofloxacin, tetracycline doxycycline, chloramphenicol, erythromycin, cotri-moxazole, ceftriaxone, cefuroxime and ampicillin. All the vibrio strains were susceptible to tetracycline (100%) and ciprofloxacin (100%). Strains were 97.5% susceptible to doxycycline, 82.5% susceptible to cefuroxime and 65% susceptible to chloramphenicol. Strains were resistant to ampicillin (90%), Cotrimoxazole (95%), ceftriaxone (75%) and 95% intermediate resistance to erythromycin.

Resistance to doxycycline was demonstrated by one isolate representing 2.5% of the 40 isolates. This implies that ciprofloxacin, tetracycline and doxycycline are the best drug of choice for the treatment of *Vibrio cholerae* nowadays even though earlier reports by Ismail et al in South Africa showed *Vibrio cholerae* resistant to tetracycline. In their study, they revealed that all 716 *Vibrio cholerae* isolates were susceptible to ciprofloxacin, ampicillin chloramphenicol, erythromycin ceftriaxone and tetracycline at the beginning of the study. However few months into the study, resistance was seen in ampicillin (2.2%), chloramphenicol (41.6%), erythromycin (24.6%) tetracycline (2.2%) and ceftriaxone (1%) but isolates were susceptible to ciprofloxacin and erythromycin. They also demonstrated the presence CT and TCP genes required for virulence and SXT integrase gene in 90 strains that were tested for by PCR. Contrary to this study and that of Ismail et al, a study by Eibach et al in 2016 demonstrates resistance of *Vibrio cholerae* to ciprofloxacin (98.4%). They found

Vibrio cholerae strains to be resistant to SXT (96.8%), ampicillin (95%) and ciprofloxacin (98.4%) in 2014, SXT (83.3%), ampicillin (91.7%) and ciprofloxacin (91.7%) in 2012, SXT (100%), ampicicillin (0%) and ciprofloxacin (0%) in 2011(Eibach et al., 2016). Rajapara et al (2015) also found in their study that all strains of *Vibrio cholerae* isolates were resistant to Cotrimoxazole, but showed intermediate resistance to ampicillin, ciprofloxacin and tetracycline. Klontz et al(2014) also demonstrated from their long term comparism of antibiotic resistance in *Vibrio cholerae* in (2000-2012) that *Vibrio cholerae* was uniformly susceptible to tetracycline until 2004-2005 when resistance level abruptly increased (Klontz et al., 2014). They also showed that resistant to erythromycin also arose at that same time (Klontz et al., 2014).

Another study by Oyedeji et al in Nigeria in the year 2013 found that *Vibrio cholerae* strains were resistant to doxycycline (32%) and tetracycline (42%). On the other hand, the 2014/2015 strains of *Vibrio cholerae* from Ghana showed all strains tested were susceptible to tetracycline and 97.5% susceptible to doxycycline. According to Oyedeji et al, strains of *Vibrio cholerae* from both Nigeria and Haiti were all resistant to Cotrimoxazole, results similar to what was obtained in the 2014/2015 isolates from Ghana.

Mahmud et al from Sierra Leone in 2014 found that all 15 *Vibrio cholerae* isolates they tested were susceptible to tetracycline, doxycycline and ciprofloxacin but found all the strains to be resistant to erythromycin, chloramphenicol and trimethoprim/sulfamethoxazole (Mahmud et al., 2014). In another study by Bhattacharya et al (2015), all four *Vibrio cholerae* isolates were sensitive to tetracycline but exhibited moderate levels of resistance to chloramphenicol. Cotrimoxazole (trimethoprim/sulfamethoxazole) is an alternative drug recommended by International Centre for Diarrhoeal Disease Research, Bangladesh and Medicins Sans Frontiers (CDC, 2015) for the treatment of the disease cholera. But recent and earlier studies

have shown resistance of *Vibrio cholerae* to the above mention drug. An earlier study by Opintan et al (2008) in Ghana reported resistance to trimethoprim/sulfamethoxazole but susceptible to quinolones and tetracycline. In their study 24 out of the 27 *Vibrio cholerae* strains were susceptible to tetracycline whiles 26 of the strains were resistant to trimethoprim/ sulfamethoxazole. This current study also reports susceptibility to tetracycline but resistance to Cotrimoxazole (trimethoprim/ sulfamethoxa-zole). However in this study, all isolates were susceptible to tetracycline. Dixit et al in 2014 also found *Vibrio cholerae* isolates being resistant to trimethoprim/ sulfamethoxazole (cotri-moxazole). They also found all 28 vibrio strains to be susceptible to tetracycline, ampicillin erythromycin and ciprofloxacin. On the contrary, this study found vibrio strains to be susceptible only to ciprofloxacin, tetracycline but resistance to Cotrimoxazole, ampicillin and erythromycin. Another study by Talkington et al (2011) from Haiti, demonstrated that all 149 *Vibrio cholerae* strains were resistant trimethoprim/sulfamethoxazole (Talkington et al., 2011). Strains were susceptible to tetracycline and either susceptible or intermediate susceptible to ampicillin and chloramphenicol. Kutar et al in 2013 also showed resistance of *Vibrio cholerae* to Cotrimoxazole (99.2%), ciprofloxacin (12.6%) and also indicated the presence of SXT element in 117 out of the 119 *Vibrio cholerae* isolates. Although tetracycline resistance has been reported in earlier studies by Chomvarin et al (2013) in Thailand and in Nigeria by Oyedeji et al in 2010, this current study however found no resistance to tetracycline and ciprofloxacin and low level of resistance to doxycycline. It therefore provides a better assurance for their usage in treating cholera. However, trimethoprim/sulfamethoxazole, ampicillin, erythromycin and ceftriaxone should not be used for the treatment of cholera since there is still resistance as high as 90% in Ghana. Susceptibility test need to be done on

chloramphenicol and cefuroxime before being given to patients since resistance to this drugs is not very high.

Integrative and conjugative elements (ICEs) form a class of mobile genetic elements that are able to encode many properties including drug resistance in many bacteria. The SXT element is an ICE that contributes to horizontal transmission and rearrangement of resistant genes in *Vibrio cholerae* (Waldor et al., 1996). A PCR detection of internal fragment of the integrase SXT element was performed on the *Vibrio cholerae* O1 isolates. SXT element was demonstrated in 39(97.5%) of the 40 isolates tested. In the study of Ramachandran et al using the same primer pair, they demonstrated SXT element in 97.2% of *Vibrio cholerae* O1, 59.7% of *Vibrio cholerae* O139 and 12.5% of non O1 and non O139 from different parts of India (Ramachandran, Bhanumathi, & Singh, 2007). Using a different primer pair set, Ceccarelli et al demonstrated SXT element in six *Vibrio cholerae* out of 13 isolates received from different parts of Angola (Ceccarelli et al., 2006). Pande et al in 2012 also demonstrated the presence of SXT element in 96.6% of isolates and also showed SXT element was not only found in Ogawa serotype but also Inaba. In recent studies, Dixit et al
2014 demonstrated the presence of SXT element in all 28 samples whiles Rajpara et al., 2015 also demonstrated SXT element in the *Vibrio cholerae* isolate they obtained from a diarrhoea patient in India. Acquisition of SXT element carrying multi drug resistance genes or markers has been shown to be attributed to multi drug resistance in *vibrio cholerae* (Waldor et al., 1996). The presence of SXT element in 39 of the 40 *Vibrio cholerae* isolates presumably is carrying multi drug resistance marker genes in their genome. This possibly accounts for the high levels of resistance seen in the antimicrobial susceptibility pattern.

Pulse field gel electrophoresis (PFGE) patterns of eight (8) isolates produced a very good pattern and were captured into Bio Numeric software for dendrogram analysis. The eight

isolates clustered together with the same pattern and shared a pattern previously seen in isolates from DR Congo, Togo, Cameroun and Ivory Coast which implies the same *Vibrio cholerae* strain has been circulating in Ghana and its neighboring countries over the years since cholera came to Ghana in the seventh pandemic

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Vibrio cholerae was isolated from 40 out of 62 rectal swabs giving a prevalence of 64.5%. The *Vibrio cholerae* isolated were serogroup O1 serotype Ogawa. In this study it was the same serotype that circulated in the 2014 cholera outbreak in Ghana. It was found that 39 *Vibrio cholerae* strains carried the CtxAB and TCP genes that codes for virulence.

The antimicrobial susceptibility of the isolates indicates resistance to Ampicillin, Cotrimoxazole, Erythromycin and Ceftriaxone but were susceptible to Ciprofloxacin, Tetracycline Doxycycline. The SXT element was highly prevalent among the isolates studied.

The PFGE of the *Vibrio cholerae* isolates cluster with isolates in DRC, Togo, Cameroun and Ivory Coast. This suggest that it is probably the same strains which are circulating in Africa.

5.2 Recommendation

It is recommended that larger number of isolates be collected across the country Ghana and studied to give a larger reflection of the vibrio strains as there were only 40 isolates that were characterized by the PFGE

The current study shows low levels of resistance to tetracycline (0%) ciprofloxacin (0%) and doxycycline (2.5%). It therefore provides a better assurance in its usage for treating cholera in Ghana. There is also the need to continuously monitor the resistance profile of *Vibrio cholerae* to antibiotics. Future studies should investigate the relationship between SXT element and the genes that encodes resistance to antibiotics. The unique fingerprint will help map sources of *Vibrio cholerae* in future outbreaks as well as to investigate the relatedness of isolates from different sources.

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WHO Situation Report on Cholera Outbreak in Ghana As of 2nd November, 2014 (Week 44)

WHO Situation Report on Cholera Outbreak in Ghana As of 20 September 2015 (Week 38)

WHO Prevention and control of cholera outbreaks: WHO policy and recommendations (2016)

APPENDIX

- **SOLID MEDIA**

TCBS

Composition

Yeast extract 5.0g, Bacteriological peptone 10.0g, Sodium thiosulphate 10.0g, Sodium citrate 10.0g, OX bile 8.0g, Sucrose 20.0g, Sodium chloride 10.0g, Ferric citrate 1.0g, Bromothymol blue 0.04g, thymol blue 0.04g, Agar 14.0g, PH 8.6±0.2

Principles

TCBS. Agar is used for selective isolation of *vibrio cholerae* and other enteropathogenic vibrio. Thiosulfate and sodium citrate as well as the alkalinity of the medium inhibits the growth of enterobacteria. OX bile and sodium cholate slows the growth of enterococci and inhibits the developments of gram positive bacteria. Fermentation of the sucrose by vibrio makes the bromothymol blue turn yellow. Bromothymol blue and thymol blue are PH indicators. Yeast extract and peptone provides the nitrogen, vitamins and amino acids in TCB agar. Sodium chloride provide optimum and metabolic activity of halophilic vibrio spp. Agar is a solidifying agent.

Preparation

Suspend 88g of agar in 1liter of distilled water. Boil to dissolve completely without autoclaving. Pour into plates and allow to solidify for further uses.

TRYPTOSE SOY AGAR

Composition

Pancreatic digest of casein 15.0g, Enzymatic digest of soya bean 5.0g, Sodium Chloride 5.0g, Agar15.0g, PH 7.3 ± 0.2

Mode of Action

Casein and soy peptone provide nitrogen, amino acids and peptides necessary for the growth of a wide variety of organisms. Sodium chloride supplies essential electrolytes and maintains osmotic equilibrium. TSA may be supplemented with blood to facilitate the growth of more fastidious bacteria.

Preparation

Add 40g to 1 liter of distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121^{o}C for 15 minutes.

- **MULLER HINTON AGAR**

Composition

Beef, dehydrate infusion 300.0g, Casein hydrolysate 17.5g, Starch 1.5g, Agar 17.0g, PH 7.3± 0.1

Mode of action

Beef extract and acid hydrolysate of casein provides nitrogen, vitamins, carbon, amino acids sulphur and other essential nutrients. Starch is added to absorb any toxic metabolites produced starch hydrolysis yields dextrose, which serves as a source of energy. Agar is the solidifying agent.

Preparation

Suspend 38gof Agar into 1 liter of distilled water. Bring to boil to dissolve completely. Sterilize by autoclaving at 121^{o}C for 15 minutes.

- **Cary-Blair Transport Medium**

Composition

Disodium hydrogen phosphate 1.1g, Sodium thioglycollate 1.5g, Sodium Chloride 5.0g, Calcium Chloride 0.09g, Agar 5.6g, Ph 8.4 ± 0.2

Mode of action

Carry -Blair medium base is prepared with minimal nutrients to facilitate survival of orgasms without multiplication. Sodium thioglycollate provides a low oxidation-reduction potential. Alkaline PH of the medium minimizes bacterial destruction due to the formation of acid. Disodium phosphate buffers the medium whereas sodium chloride maintains the osmotic equilibrium.

Preparation

Suspend 13.3g in 1litre of distilled water and bring gently to boil to dissolve the ager. Distribute into small screw cap bottles and sterilize by immersing in free stream for 15 minutes. Allow to cool and tighten the screw caps to prevent water loss.

- **LIQUID MEDIA**

Alkaline peptone water

Composition

Peptone 10.0g, Sodium Chloride 20.0, PH8.6 ± 0.2

Mode of action

Alkaline peptone water is for the enrichment of *vibrio cholera*e and vibrio species from food, water, and clinical samples. The sodium chloride incorporated in the medium promotes the

growth of *vibrio cholerae* while the alkalinity of this medium inhibits most of the unwanted flora.

Preparation

Suspend 30g of Agar to 1 liter of distilled water, mix well, distribute into final containers and sterilize by autoclaving at 121^0C for 15 minutes.

- **PCR PROTOCOL**

- Pippete 33.75ul of nuclease free water into 1.5uleppendorftube
- Add 1 ul of forward primer
- Add 1 ul of reverse primer
- Add 4 ul of magnesium chloride
- Add 5 ul of X1O buffer
- Pippete 46ul of master mix into 0.1ul tubes to be use for PCR amplification
- Add 2ul of DNA template into the 0.1 ul tube
- Place the tubes in the thermocycler making sure there are no bubbles. Start to run the PCR

- **PFGE PROTOCOL**

CASTING OF PLUGS

- Transfer 400 ul (0.4ml) adjusted cell suspension to labeled 1.5 microcentrifuge tubes
- Add 20 ul of proteinase k (20 mg/ml stock) to each tube and mix gently with pipet tip
- Add 400 ul (0.4) melted 1% Seakem Gold Agarose to the 0.4-ml cell suspension; mix by gently pipetting the mixture up and down a few times. Maintain temperature of melted agarose by keeping flask in beaker of warm water (55-60^{o}C)

- Immediately, dispense part of mixture into appropriate wells of reusable plug molds without bubbles forming. Allow plugs to solidify at room temperature for 10-15 minutes. They can also be placed in the refrigerator (4^{o}C) for 5 minutes.

LYSES OF CELL IN AGAROSE PLUGS

- Label 50ml polypropylene screw cap or 50ml Oak Ridge tubes with culture numbers
- Prepare Cell Lysis Buffer (50 mMTris: 50 mM EDTA, PH 8.0 +1% Sarcosyl)
- Dilute to 500 ml with sterile ultrapure water (CLRW)
- Calculate the total volume of cell lysis / proteinase k buffer needed
- Add 5ml of proteinase K/Cell lysis Buffer to each labeled 50 ml tube
- Trim excess agarose from top of plugs with scalpel, or razor blade. Open reusable plug mold and transfer plugs from mold with 6mm wide spatula to appropriately labeled tube. If disposable plug molds are used, remove white tape from bottom of mold and push out plug(s) into appropriately labeled tube. Making sure plugs are under buffer and not on side of tube.
- Remove tape from reusable mold. And disinfect all apparatus used in 70% isopropanol or ethanol for 15 minutes before washing them
- Place tubes in racks and incubate in 54-55^{o}C shaker water bath or incubator for 1.52hrs with constant and vigorous agitation (150-175rpm). If lysing in water bath, be sure water level is above level of lysis buffer in tubes.
- Pre- heat enough sterile Ultrapure water to 54-55^{o}C so that plugs can be washed two times with 10-15 ml water.

WASHING OF AGAROSE PLUGS AFTER CELL LYSIS

- Remove tubes from water bath or incubator, and carefully pour off lysis buffer into an appropriate discard container; plugs can be held in tubes with a screened cap or spatula.
- Add 10-15 ml sterile Ultrapure water that has been pre-heated to 54-55^{o}C to each tube and shake the tubes in a 54-55^{o}C water bath or incubator for 10-15 minutes.
- Pour off water from the plugs and repeat wash step with pre-heated water one more time. Pre-heat enough sterile TE (10mM Tris; 1mM EDTA, PH 8.0) in a 54-55^{o}C water bath so that plugs can be washed four times with 10-15 ml TE
- Pour off water, add 10-15ml pre-heated TE buffer, and shake the tubes in 54-55oC water bath or incubator for 10-15 minutes
- Pouf off TE and repeat wash step with pre-heated TE three more times.
- Decant last wash and add 5-10ml sterile TE. Plugs can be transferred to smaller tubes for long term storage.

LOADING OF GEL.

- Confirm that water bath is equilibrated to 55-60^{o}C
- Make volume of 0.5X Tris -Borate EDTA Buffer (TBE) that is needed for both the gel and electrophoresis.
- Make 1% Seakem Gold (SKG) agarose in 0.5X TBE
- A small volume of (2-5ml) melted and cooled (55-60^{0}C) 1% SKG agarose may be wanted to seal wells after plugs are loaded
- Remove restricted plug slices from37^{o}C water bath. Remove enzyme/buffer mixture and add 200ul 0.5X TBE. Incubate at room temperature for 5 minutes
- Remove plug slices from tubes and load plug slices on the bottom of the com bteeth.

- Load S. SerBraenderup H9812 standards on teeth (lanes 1,5,10)
- Load samples on remaining teeth
- Remove excess buffer with tissue and allow plug slices to air dry on the comb for 3-5 minutes or seal them to the comb with 1% SKG agarose (55-60^{o}C)
- Position comb in gel form and confirm that the plugs slices are correctly aligned on the bottom of the comb teeth that the lower edge of the plugs slice is flush against the black platform and there are no bubbles.
- Carefully pour the agarose (cooled to 55-60^{o}C) into the gel form.
- Put back gel frame in electrophoresis chamber and close cover of the unit,
- Turn on module (14^{o}C) power supply and pump (set at 70 to achieve a flow rate at 1 liter/minute)
- Remove comb after gel solidifies for 30-45 minutes
- Fill in wells of gel with melted and cooled (55-60^{0}C) 1% SKG Agarose. Unscrew and remove end gates from gel form; remove excess agarose from slides and bottom of casting platform with tissue. Keep gel on casting platform and carefully place gel inside black gel frame in electrophoresis chamber. Close cover of chamber.

ELECTROTHROPHORE CONDITIONS

Auto -Algorithm

30kb - low mw

700kb – high mw

Select default values except where noted by pressing “enter” Change

run time to 18-19h.

(Default values: initial switch time=2.16s, Final switch time=63.8s)

STAINING AND DOCUMENTARY OF PFGE AGAROSE GEL.

- When electrophoresis run is over, turn off equipment. Remove and stain gel with ethidium bromide. Dilute 40 ml of ethidium bromide stock solution (10mg/ml) with 400 ml of reagent grade water. Strain gel for 20-30 min in covered container
- -Destain gel in approximately 500ml reagent grade water for 60-90 minutes. Change water every 20 minutes. Capture image on a Gel Doc 1000, 2000, EQ or XR or equivalent documentation system.
- Follow directions given with the imaging equipment to save the gel image as an *.img or *.isc file convert this file to *tif file for analysis with Bionumerical Software Program.
- Drain buffer from electrophoresis chamber and discard.